Calcium-Dependent Protein Kinase Isoforms in Petunia Have Distinct Functions in Pollen Tube Growth, Including Regulating Polarity

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Calcium is a key regulator of pollen tube growth, but little is known concerning the downstream components of the signaling pathways involved. We identified two pollen-expressed calmodulin-like domain protein kinases from Petunia inflata, CALMODULIN-LIKE DOMAIN PROTEIN KINASE1 (Pi CDPK1) and Pi CDPK2. Transient overexpression or expression of catalytically modified Pi CDPK1 disrupted pollen tube growth polarity, whereas expression of Pi CDPK2 constructs inhibited tube growth but not polarity. Pi CDPK1 exhibited plasma membrane localization most likely mediated by acylation, and we present evidence that suggests this localization is critical to the biological function of this kinase. Pi CDPK2 substantially localized to as yet unidentified internal membrane compartments, and this localization was again, at least partially, mediated by acylation. In contrast with Pi CDPK1, altering the localization of Pi CDPK2 did not noticeably alter the effect of overexpressing this isoform on pollen tube growth. Ca2+ requirements for Pi CDPK1 activation correlated closely with Ca2+ concentrations measured in the growth zone at the pollen tube apex. Interestingly, loss of polarity associated with overexpression of Pi CDPK1 was associated with elevated cytosolic Ca2+ throughout the bulging tube tip, suggesting that Pi CDPK1 may participate in maintaining Ca2+ homeostasis. These results are discussed in relation to previous models for Ca2+ regulation of pollen tube growth.

INTRODUCTION

After a compatible pollination, pollen germinates to extrude a tube that penetrates the stigma and grows extracellularly through the stylar tissues to the ovules. These tubes serve as conduits through which the sperm cells travel to effect fertilization. Pollen tubes extend by tip growth, a process characterized by highly polarized exocytosis. As growth proceeds, these tubes exhibit vigorous tip-focused, reverse-fountain cytoplasmic streaming with rapidly moving granular elements seen behind a clear zone at the apical dome of the tube. The clear zone represents the region to which secretion is localized (Heslop-Harrison, 1987; Steer and Steer, 1989; Pierson et al., 1990).

It is well established that Ca2+ plays a critical role in regulating tip growth (Miller et al., 1992; Pierson et al., 1994; Malhó and Trewavas, 1996; Hepler, 1997; Messerli and Robinson, 1997). A steep tip-focused gradient in cytosolic free Ca2+ concentration ([Ca2+]i) is present in growing pollen tubes and absent in non-growing tubes, and its perturbation results in a reversible cessation of tip growth (Rathore et al., 1991; Pierson et al., 1994; Li et al., 1996). Furthermore, it is possible to change pollen tube growth orientation by activation of caged Ca2+ or Ca2+-ionophore to locally manipulate intracellular Ca2+ levels (Malhó et al., 1994; Bibikova et al., 1997). [Ca2+]i ranges from ~2 to 10 μM at the apex, dropping to ~20 to 200 nM within 20 μm of the tip (e.g., Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994; Franklin-Tong et al., 1997). Influx of Ca2+ is localized to a small region at the tube tip (e.g., Malhó et al., 1994, 1995; Feijó et al., 1995, 1996; Malhó and Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997), and this tip-localized entry appears largely responsible for formation of the gradient, although other mechanisms, such as inositol triphosphate-dependent release from Ca2+ stores, may also play a role (Pierson et al., 1994; Malhó et al., 1995). It has been proposed that subapical dissipation of the Ca2+ gradient is regulated by Ca2+-ATPases either on tubular endoplasmic reticulum behind the apical region (Obermeyer and Weisenseel, 1991; Lancelle and Hepler, 1992) or at the plasma membrane (Schiott et al., 2004).

The actin cytoskeleton and its interaction with signaling pathways also play a major role in pollen tube tip growth (Steer and Steer, 1989; Derksen et al., 1995; Hepler et al., 2001). Dynamic cortical actin cables are abundant in the tube shank and extend to the base of the clear zone (Geitmann et al., 2000; Vidalí et al., 2001; Chen et al., 2002). Recent evidence suggests that the clear zone itself possesses a dense cortical fringe of longitudinal actin filaments starting 1 to 5 μm behind the tip and extending basally for 5 to 10 μm (Lový-Wheeler et al., 2005). Actin microfilaments...
are involved in cytoplasmic streaming and vesicle transport but may play additional roles in growth. For example, tube elongation ceases when actin polymerization is inhibited under conditions where there is no loss of cytoplasmic streaming (Vidali et al., 2001). Microfilament dynamics are regulated by actin binding proteins, including profilin, actin-depolymerizing factors (ADFs)/cofilins, and gelsolin/villins (Staiger et al., 1997; McGough, 1998; Bamburg, 1999; Hepler et al., 2001; Higgs and Pollard, 2001; Huang et al., 2004). A pollen-specific ADF has been identified that associates with the subapical mesh of actin filaments at the pollen tube tip. Overexpression of this protein leads to growth inhibition (Chen et al., 2002, 2003). Ca2+ is also known to regulate actin dynamics (Blancaflor, 2002), suggesting a regulatory loop where the tip-focused Ca2+ gradient may regulate the cytoskeletal apparatus driving growth.

Rop/Rac GTPases also appear to be central regulators of pollen tube growth (Lin and Yang, 1997; Kost et al., 1999; Li et al., 1999). Experiments employing overexpression and catalytically modified forms of Rop/Rac have demonstrated that some members of this family of GTPases are critical for tip growth (Kost et al., 1999; Li et al., 1999). Overexpression of constitutively active Rop/Rac (which irreversibly binds GTP) promotes pollen germination but does so in a depolarized manner, resulting in bulbous or swollen pollen tubes (as does Rop/Rac overexpression, albeit to a lesser extent; Li et al., 1999). Plasma membrane localization is essential for the biological function of these GTPases and is mediated by isoprenylation (Lin et al., 1996; Kost et al., 1999; Li et al., 1999). Multiple downstream effectors appear to transduce Rop/Rac activity. A number of Rop-interactive CRIB-containing proteins (RICs) have been identified, and two (RIC3 and RIC4) have recently been shown to play opposing roles in regulating actin dynamics (Gu et al., 2005). Overexpression of a pollen-specific ADF rescues loss of growth polarity caused by overexpressing Rop/Rac and has been proposed to be another downstream component of Rop/Rac signaling pathways (Chen et al., 2003). Evidence also suggests that Rop/Rac acts upstream of Ca2+ in tip growth (Li et al., 1999), in which case this protein may regulate the tip-localized influx of Ca2+ and formation of the Ca2+ gradient, leading to the activation of growth (Zheng and Yang, 2000).

While it is clear that a tip-focused Ca2+ gradient is essential for tip growth, and actin and Rop/Rac GTPases are key components of the tip growth machinery, little is known concerning the identity of downstream effectors of Ca2+ in this process or precisely how Ca2+ dynamics modulate the tip growth machinery. One class of likely candidates for such modulators is the calcium-dependent protein kinases (CDPKs). CDPK activity localizes with F actin in pollen (Putnam-Evans et al., 1989). Antisense oligonucleotides of a maize (Zea mays) pollen-specific CDPK impair pollen germination and tube growth, as does addition of kinase inhibitors and calmodulin antagonists (Estruh et al., 1994). Localization of a CDPK-like activity in the tips of pollen tubes has been inferred by imaging with a fluorescent substrate of protein kinase C (PKC). Using this approach, redirection of the tip-focused Ca2+ gradient via asymmetric photolysis of caged Ca2+ was found to be associated with an increase in kinase activity in the region of Ca2+ release (Moutinho et al., 1998). These data are consistent with a CDPK acting as a Ca2+ response element at the tip of the growing tube.

In this study, we set out to identify CDPK isoforms expressed in pollen of Petunia inflata and to characterize them and define their functions in pollen tube growth. We have identified two CDPK isoforms, which are highly expressed in pollen tubes. We report experiments that suggest that both play major roles in pollen tube growth but that they have divergent localizations and functions. One appears to be a key regulator of growth polarity, whereas the second is more generally involved in pollen tube extension.

RESULTS

Cloning of CDPK Isoforms Expressed in Pollen

To identify pollen-expressed CDPK isoforms, we performed 3′ rapid amplification of cDNA ends (RACE) PCR using a degenerate primer (CDPK1) based on the highly conserved amino acid sequence VPPFWA present in the catalytic domain of CDPKs (McCubbin et al., 2004). Pollen cDNA was synthesized using an oligo(dT) adaptor and amplified using CDPK1 and the adaptor primer, resulting in a diffuse band of the predicted size (1.1 kb). This band was cloned, and restriction analysis of 20 clones revealed two classes in approximately equal proportions. Sequencing revealed that both classes encoded CDPK isoforms, and they were named Pi CDPK1 and Pi CDPK2. Full-length cDNAs were obtained for each class by screening a pollen cDNA library. At low stringency, individual probes hybridized to both cDNA classes; however, no additional classes were identified in screening 2 × 106 clones, suggesting that these are the predominant CDPK isoforms expressed in Petunia pollen. Data gathered by probing whole Arabidopsis thaliana genome gene chips with pollen cDNA suggest that 16 of the 34 CDPK isoforms in Arabidopsis are expressed above background levels in pollen, and five (Calmodulin-domain protein kinases [CPKs] 14, 16, 17, 24, and 34) are expressed at high levels (Harper et al., 2004). There are two possibilities why we did not find additional pollen-expressed isoforms in Petunia. The CDPK gene family may be smaller in Petunia than in Arabidopsis, or isoforms expressed at low levels might be underrepresented in our cDNA libraries. RNA gel blot analysis of Pi CDPK1 and Pi CDPK2 indicates that both are highly expressed in pollen and are pollen predominant and possibly specific, though expression in cell types underrepresented in this analysis (including other tip-growing cells, such as root hairs) remains a possibility. Expression of each isoform commences in 15- to 20-mm flower buds (at which point the microspores have just completed pollen mitosis I), peaks in mature pollen, and remains high after germination (Figures 1A and 1B).

CDPKs possess an N-terminal variable domain and three other fairly well-conserved domains: a kinase catalytic domain, an autoinhibitory domain, and a C-terminal calmodulin-like domain (for review, see Hrabak, 2000). Analysis of predicted amino acid sequences demonstrated that both isoforms contain all four domains. Pi CDPK1 is most similar to Arabidopsis CPK17 (GenBank accession number 9759385), with which it shares 78% amino acid identity. Pi CDPK1 also shares 77% amino acid identity with a previously identified pollen-expressed CDPK from
maize (Estruch et al., 1994). Pi CDPK2 is most similar to Arabidopsis CDPK24 (GenBank accession number 4582467), with which it shares 65% identity at the amino acid level. Both CDPK17 and CDPK24 are highly expressed in Arabidopsis pollen (Harper et al., 2004), suggesting that homologs of these isoforms are likely to be abundant in pollen in a broad range of species. Full-length Pi CDPK1 and Pi CDPK2 are 46.9% identical to each other at the amino acid level (21.1% for the N-terminal variable domains, 61.1% for the kinase domains, 38.2% for the autoinhibitory domains, and 35.6% for the calmodulin-like domains). This sequence divergence, along with the fact that CDPK17 and CDPK24 are widely separated on a phylogenetic tree of all Arabidopsis isoforms (Hrabak, 2000), suggested to us that the two isoforms might have divergent functions in pollen tubes. Therefore, we next sought to define the roles of these kinases in pollen tube growth.

Effects of Transiently Expressing Wild-Type or Mutant CDPK Isoforms in Pollen Tubes

Both Pi CDPK1 and Pi CDPK2 are highly expressed prior to pollen maturity (Figure 1). As we were interested in the roles of these isoforms in tube growth rather than pollen development, and a pollen tube–specific promoter has not yet been identified, we employed a transient transformation system to avoid potentially deleterious effects on pollen development that would be intrinsic to stably transformed plants expressing mutated versions of these proteins. We have previously used transient expression of catalytically modified CDPK constructs to study the role of a CDPK isoform in the gibberellic acid response in barley (Hordeum vulgare) aleurone and have shown that a catalytically inactive mutant CDPK exerts a dominant-negative effect (McCubbin et al., 2004). We therefore developed a similar strategy employing overexpression, catalytically inactive, and constitutively active constructs to investigate the functions of Pi CDPK1 and Pi CDPK2 in pollen tube growth.

Dominant-negative (DN) constructs were generated by point mutating the catalytic site of each CDPK from VIHRDLK to VIHRNLK. We have previously shown that this mutation caused a >100-fold reduction in the kinase activity of Hv CDPK1 (McCubbin et al., 2004). Constitutively active (CA) versions of each isoform were generated by truncating each at the junction between the kinase and autoinhibitory domains (corresponding to Glu-334 in Pi CDPK1 and Asn-320 in Pi CDPK2). Equivalent truncation previously has been shown to liberate the resultant enzyme from calcium dependency (Harper et al., 1994). C-terminal green fluorescent protein (GFP) fusions were also generated for all mutant and wild-type constructs.

To confirm that the constructs possessed the catalytic activities predicted, wild-type and mutant versions of Pi CDPK1 were expressed in Escherichia coli as His-tagged fusion proteins, purified, and assayed for calcium-regulated protein kinase activity following the method of Ritchie and Gilroy (1998). The kinase activity of the DN mutant was reduced >50-fold relative to the wild-type protein regardless of calcium concentration, and the CA mutant protein exhibited calcium-independent kinase activity (~60% of the maximum activity exhibited by the wild-type protein; see Supplemental Table 1 online). Furthermore, the GFP fusions exhibited the same catalytic characteristics as their respective nonfusion proteins (see Supplemental Table 1 online).

For each CDPK isoform, wild-type (overexpression), DN, and CA constructs were generated under control of the Lat52 promoter, which was selected because it promotes gene expression in a manner almost indistinguishable to native expression patterns of the two CDPKs (Figure 1; Twell et al., 1989). In agreement with many other studies (e.g., Li et al., 1999; Chen et al., 2002; Cheung et al., 2003), we observed that control transformations using ProLat52::GFP showed that these tubes were able to grow for >4 h in liquid culture, expressing GFP but with no other discernable phenotype. This control indicated that neither bombardment nor GFP expression altered growth characteristics (Figures 2G and 3G). Nonfusion constructs were bombarded into pollen with ProLat52::GFP as a marker for transformation, whereas GFP fusion constructs were bombarded alone. To determine consensus phenotypes, 100 individual transformants were scored for each construct from three independent bombardments. In all cases, phenotypes generated were highly reproducible between bombardments, and those generated by nonfusion constructs were indistinguishable from their respective GFP fusions.

Effects of Pi CDPK1 Constructs on Pollen Tube Growth

Overexpression of Pi CDPK1 and Pi CDPK1-GFP both caused a dramatic loss of growth polarity in transformed tubes, resulting in extremely short tubes with almost spherical tips (pollen tubes transformed with Pi CDPK1-GFP are shown in Figures 2A and 2B). This phenotype was exhibited by 82% of transformants overexpressing Pi CDPK1 (as judged by ballooning of the tube to a diameter equal to or greater than that of the pollen grains).
Expression of Pi CDPK1/DN also caused loss of growth polarity, but less severely than the wild-type protein, with transformed tubes apparently losing polarity more gradually, resulting in swollen sock-like tubes in 86% of transformants (Figures 2C and 2D).

Expression of the unregulated kinase domain of Pi CDPK1, the kinase activity of which is not constrained by \([\text{Ca}^{2+}]_i\), severely inhibited both pollen germination and tube growth (both length and width). As opposed to 12% in samples bombarded with ProLat52:Pi CDPK1-GFP, 43% of the pollen failed to germinate. This was the only construct that had a significant effect upon pollen germination. The 46% of pollen that did germinate exhibited a substantial reduction in tube width and failed to extend more than one pollen grain diameter (Figures 2E and 2F). For comparison, a representative pollen tube from a control transformation with ProLat52:GFP is shown in Figure 2G. Bars = 20 \(\mu\)m.

**Effects of Pi CDPK2 Constructs on Pollen Tube Growth**

Phenotypes generated by expression of Pi CDPK2 constructs in pollen were distinct from those of Pi CDPK1 (Figures 3A to 3F). Expression of all Pi CDPK2 constructs led to inhibition of pollen tube extension but did not affect growth polarity or germination rates, resulting in short tubes with essentially normal morphology. Overexpression of Pi CDPK2 caused an average of 41% reduction in tube length (SD = 14.0%, \(n = 100\)), Pi CDPK2/DN an average 35% reduction in tube length (SD = 14.1%, \(n = 100\)), and Pi CDPK2/CA an average 57% reduction in tube length (SD = 8.1%, \(n = 100\)). Student’s \(t\) tests indicated the level of significance to be \(P < 0.0001\) for all three constructs when compared with controls (Figure 3G).

**Localization of Pi CDPK1 and Pi CDPK2**

CDPK isoforms localize to a variety of intracellular locations, including the cytosol, nucleus, plasma membrane, endoplasmic reticulum, peroxisomes, mitochondrial outer membrane, and oil bodies (Pical et al., 1993; Patharkar and Cushman, 2000; Lu and Hrabak, 2002; Anil et al., 2003; Dammann et al., 2003). We employed confocal microscopy to assess the subcellular localization of each CDPK isoform using full-length C-terminal GFP fusions. Pi CDPK1-GFP localized mainly to a thin layer at the periphery of the pollen tube, consistent with a plasma membrane location, though some fluorescence was also visible in the cytoplasm (Figure 4A). To date, this is the most common localization pattern found for CDPK isoforms (Dammann et al., 2003). By contrast, Pi CDPK2-GFP labeled internal membrane compartments (Figure 4E); the precise nature of these compartments...
is currently unknown and is under investigation, but it is clear that Pi CDPK2 does not localize to the plasma membrane and that its localization differs from that of Pi CDPK1.

**Relationship of Pi CDPK1 and Pi CDPK2 Localization to Their Functions**

Neither CDPK isoform possesses transmembrane domains; however, like most CDPKs, they possess putative N-terminal acylation sites. Each has a Gly residue at position 2 (potential myristoylation site) followed by Cys residues at positions 4 and 5 in Pi CDPK1 and position 4 in Pi CDPK2 (potential palmitoylation sites). Lipid modifications have been shown to regulate CDPK localization (Martínez and Busconi, 2000) and have been predicted to be important to their biological functions (Dammann et al., 2003; Chehab et al., 2004). The observation that both Pi CDPK1 and Pi CDPK2 localize to membranes suggests that at least one of these modifications occurs in vivo for these kinases.

To confirm that the localization of Pi CDPK1 and Pi CDPK2 is a result of N-terminal acylation and investigate whether localization is important to their biological function(s), we generated pollen expression constructs in which the N-terminal six amino acids were deleted ($\Delta N$–Pi CDPK1 and $\Delta N$–Pi CDPK2). These deletions remove both putative palmitoylation and myristoylation sites and are predicted to prevent the membrane localizations exhibited by the full-length proteins if such localization were directed by N-terminal acylation. It has been shown previously that deletion of the entire N-terminal variable domain of a CDPK does not affect either kinase activity or calcium dependency (Harper et al., 1994); hence, these small deletions are unlikely to affect these properties. This was confirmed for $\Delta N$–Pi CDPK1 (see Supplemental Table 1 online), but technical difficulties in expressing the protein in *E. coli* precluded this analysis for $\Delta N$–Pi CDPK2.

$\Delta N$–Pi CDPK1-GFP is visible only in the cytosol, rather than showing the plasma membrane localization exhibited by the full-length protein (Figure 4B). Importantly, in contrast with tubes expressing full-length Pi CDPK1, pollen tubes expressing this truncated protein grew in a normal polar manner (Figures 4C and 4D). As $\Delta N$–Pi CDPK1-GFP and Pi CDPK1-GFP differ only in the N-terminal six-amino acid deletion, this result suggests that plasma membrane localization of Pi CDPK1 is essential to its function in the regulation of growth polarity.

$\Delta N$–Pi CDPK2-GFP fluorescence was also found largely in the cytosol, though weak internal membrane localization was detectable (Figure 4F). The substantial loss of internal membrane association suggests that Pi CDPK2 also undergoes lipid modification(s) that mediates subcellular targeting. In contrast with

*Figure 3. Representative Pollen Tubes Expressing Pi CDPK2 Constructs.*

Images show pollen cultured in vitro for 4 h after biolistic bombardment. Paired images show GFP fluorescence micrographs on a black background (showing transformed tubes), alongside equivalent light micrographs on a gray background (showing both transformed and wild-type tubes). Tube growth was inhibited by all Pi CDPK2 constructs, but growth polarity was not affected: Pro$_{lat52}$-Pi CDPK2-GFP ([A] and [B]); Pro$_{lat52}$-Pi CDPK2/DN-GFP ([C] and [D]); Pro$_{lat52}$-Pi CDPK2/CA-GFP ([E] and [F]). A control transformant expressing Pro$_{lat52}$:GFP is shown for comparison (G). Bars = 20 µm.
the lack of an effect on tube growth of \( \Delta N \)-Pi CDPK1-GFP, pollen tubes expressing \( \Delta N \)-Pi CDPK2-GFP were still inhibited to an extent comparable to tubes overexpressing the wild-type protein, possibly indicating that membrane localization is not critical to the events leading to the Pi CDPK2 overexpression phenotype.

Requirements for Pi CDPK1 Activation in Relation to \([\text{Ca}^{2+}]\) in Vivo

As noted above, a tip-focused \( \text{Ca}^{2+} \) gradient is essential to pollen tube growth. As CDPKs are calcium regulated, the loss of growth polarity observed in pollen tubes overexpressing Pi CDPK1 is likely to be caused by increased Pi CDPK1 activity associated with the \([\text{Ca}^{2+}]\) gradient at the tube tip. To enable us to determine where Pi CDPK1 is active in wild-type tubes, we used in vitro assays to determine the \( \text{Ca}^{2+} \) requirements for activation of recombinant Pi CDPK1 and performed ratiometric \( \text{Ca}^{2+} \) imaging on wild-type pollen tubes to assess the \( \text{Ca}^{2+} \) environment in which these kinases operate. The concentration of \( \text{Ca}^{2+} \) required for Pi CDPK1 activation was found to correlate with the concentration of \( \text{Ca}^{2+} \) in the tip region of the pollen tube. Thus, the bulk of kinase activation occurred in the 100- to 1000-nM range, with the \( \text{Ca}^{2+} \) concentration required for half maximal activation being \( \sim 600 \) nM (Figures 5 and 6A). In a wild-type pollen tube, imaging revealed \( \text{Ca}^{2+} \) levels reaching \( \sim 1 \) \( \mu \text{M} \) in a small region at the tube tip (Figure 6C). Interestingly, in the swollen apex of tubes expressing \( \text{ProLat52}:\text{Pi CDPK1-GFP} \) (Figure 6A), \( \text{Ca}^{2+} \) levels of this magnitude were seen in a greatly expanded region. By contrast, overexpression of Pi CDPK2 did not have a discernable effect on the tip-focused \([\text{Ca}^{2+}]\) gradient (Figure 6B).

DISCUSSION

Previous studies have shown that a CDPK isoform is necessary for pollen germination and tube growth (Estruch et al., 1994) and that a calcium-dependent kinase activity is associated with pollen tube reorientation (Moutinho et al., 1998). We have shown that CDPK isoforms play distinct roles in pollen tube growth and have distinct localizations. Pi CDPK1 appears to be a critical component of a signaling pathway(s) regulating growth polarity, and plasma membrane localization appears to be key to the biological function of this kinase. These data suggest that Pi CDPK1 is likely the Petunia homolog of the enzyme responsible for the calcium-dependent kinase activity imaged by Moutinho et al. (1998), which was restricted to the plasma membrane and its immediate cytoplasmic vicinity.

Figure 5. Calcium Requirements for Pi CDPK1 Phosphorylation of Syntide-2.

Relative kinase activity is represented as a fraction of maximal activity (2042 pmol min\(^{-1}\) mg\(^{-1}\)). Results represent the mean of three separate experiments.
By contrast, our results suggest that Pi CDPK2 plays a role in pollen tube extension but not polarity. Overexpression of wild-type, DN, and CA Pi CDPK2 constructs all inhibited tube extension but did not affect growth polarity. Pi CDPK2 localizes to an as yet unidentified internal membrane system(s). Deletion of the N-terminal acylation sites of Pi CDPK2 greatly decreased but did not abolish this localization, suggesting that lipid modification(s) is an important, but not the only, factor determining this targeting within the cell. As overexpression of wild-type Pi CDPK2 and ΔN–Pi CDPK2 generated the same phenotype, membrane localization may not be critical to the biological function of this isoform. We cannot discount, however, the possibility that the small residual localization seen with ΔN–Pi CDPK2 is responsible for this maintained phenotype.

The results presented provide clues as to how Pi CDPK1 might act in the regulation of pollen tube growth polarity. Pi CDPK1 overexpression is associated with elevated [Ca^{2+}] at the tube tip. Bulging of pollen tube tips is not always associated with elevated Ca^{2+} (Cárdenas et al., 2005); hence, this result suggests that Pi CDPK1 may be involved in regulating Ca^{2+} homeostasis. Ca^{2+} has long been known to impact growth polarity in several ways. High [Ca^{2+}] stimulates exocytosis and also disrupts the actin cytoskeleton in lily (Lilium longiflorum) pollen tubes (Kohno and Shimmen, 1987). Ca^{2+} has also been implicated in vesicle transport in pollen tubes (Picton and Steer, 1985). One mechanism by which [Ca^{2+}] might affect microfilament dynamics is through one or more actin binding proteins. Profilins, gelsolin/villin, and ADFs have all been implicated in this process in pollen tubes (Kovar et al., 2000; Fu et al., 2001; Chen et al., 2002, 2003; Huang et al., 2004). A maize ADF (ADF3) has been shown to be regulated by phosphorylation by a CDPK, phosphorylation reducing the ability of ADF to depolymerize actin filaments (Smertenko et al., 1998; Allwood et al., 2001). There is also evidence that a pollen-expressed ADF (Nt ADF1) is similarly phosphoregulated, though pollen tube extracts appear not to possess CDPK activity capable of phosphorylating Nt ADF1 (Chen et al., 2002).

In animal cells, exocytosis is triggered by high [Ca^{2+}], at the plasma membrane and is regulated by PKC (Burgoyne and Morgan, 1993). In plants, high [Ca^{2+}], is also required for the aggregation, fusion, and exocytosis of secretory vesicles at the plasma membrane and may be responsible for triggering these processes (Battey and Blackbourne, 1993; Roy et al., 1999). A true PKC homolog has not been found in plant genomes, and CDPKs have been hypothesized to fulfill at least some of the functions of PKC in plants (Namnori et al., 1994; Abo-El-Saad and Wu, 1995). The results presented suggest that Pi CDPK1 is a good candidate for performing at least some PKC functions related to secretion in plants.

There are two mechanisms by which excess Pi CDPK1 activity might support elevation of [Ca^{2+}] at the tube tip: stimulation of Ca^{2+} uptake and/or inhibition of efflux. Influx of Ca^{2+} is thought to be mediated by plasma membrane Ca^{2+} channels at the tube apex (e.g., Jaffe et al., 1975; Pierson et al., 1994, 1996; Malhó et al., 1995; Messerli and Robinson, 1997). Evidence suggests that these channels are stretch activated and/or voltage gated (Feijó et al., 1995; Malhó et al., 1995; Malhó and Trewavas, 1996; Pierson et al., 1996; Dutta and Robinson, 2004; Wang et al., 2004). Gating of stretch-activated Ca^{2+} channels is well characterized as being potentiated by protein–protein interactions and posttranslational modifications in other systems (for reviews, see Mark and Herlitze, 2000; Schultz and Dubinsky, 2001; Hoenderop et al., 2002) and is a potential site of influence for Pi CDPK1. Releasing caged Ca^{2+} or Ca^{2+} ionophore stimulates prolonged [Ca^{2+}] elevation beyond that directly induced by the caged probe itself, suggesting the presence of a positive feedback mechanism where a small amount of Ca^{2+} influx may trigger a self-sustaining intracellular elevation (Malhó and Trewavas, 1996; Bibikova et al., 1997). It has been proposed that this mechanism may include a tip-localized gradient of kinase activity promoting Ca^{2+}-mediated exocytosis (Moutinho et al., 1998).

Low-level activation of PKC can potentiate [Ca^{2+}] increase by blocking the activation of Ca^{2+} efflux (Petersen and Berridge, 1994), and recent data have provided evidence that CDPKs could play a similar role (Hwang et al., 2000). The endoplasmic reticulum calmodulin-stimulated Ca^{2+} pump Arabidopsis Calcium-transporting ATPase2 (ACA2) is inhibited by phosphorylation by a CDPK (Arabidopsis CPK1; Hwang et al., 2000). Four ACAs are expressed in Arabidopsis pollen (9, 7, 11, and 2) (AtGenExpress microarray database; http://www.arabidopsis.org/info/expression/AtGenExpress.jsp). ACA9 localizes to the plasma membrane and is required for normal pollen tube growth and fertilization (Schiott et al., 2004). It is not clear whether CDPK-mediated inhibition of ACAs occurs in vivo (Hwang et al., 2000); however, the localization of Pi CDPK1 places this enzyme in prime position to participate in regulating a plasma membrane Ca^{2+} pump that helps limit Ca^{2+} elevation at the pollen tube tip.

The strategy of expressing catalytically modified CDPK constructs in vivo generated phenotypes for both isoforms. In the case of Pi CDPK2, the phenotypes generated by the overexpression, CA, and DN constructs were similar, making it difficult to speculate on the function of this isoform. One explanation...
for these results is that this isoform plays a complex role in pollen tube growth, perhaps involving multiple substrates with antagonistic functions. A possible alternative, which cannot be excluded, is that the main role of Pi CDPK2 in pollen tube growth is not related to kinase activity. For Pi CDPK1 constructs, phenotypes varied considerably, providing some insight into Pi CDPK1 function. Intriguingly, loss of growth polarity was seen in both Pi CDPK1 overexpression (predicted to lead to excess substrate phosphorylation) and Pi CDPK1-DN expression (predicted to reduce the rate of substrate phosphorylation by competing with endogenous Pi CDPK1). The latter result contrasts with our previous analysis of Hv CDPK1, where a DN construct was found to inhibit secretion associated with the gibberellic acid response in aleurone cells (McCubbin et al., 2004). This difference likely reflects functional divergence between these kinases. For example, the two enzymes exhibit different subcellular localizations, Hv CDPK1 being cytosolic, whereas Pi CDPK1 is largely plasma membrane localized. A second observation is that Pi CDPK1 overexpression and expression of the CA mutant (Pi CDPK1-CA) lead to opposing phenotypes when both are expected to lead to excess phosphorylation of substrates. These findings suggest that Pi CDPK1 may not directly regulate a molecule critical to growth polarity but rather acts through an intermediary molecule(s), possibly entraining several components of the tip growth machinery to the tip-focused Ca^{2+} gradient.

The phenotypes generated by Pi CDPK1 constructs are explicable in relation to a single substrate if the action of Pi CDPK1 is to repress the activity of a molecule (X) that negatively regulates a factor (Y), which regulates polarity. A model illustrating how such a scenario might regulate tip growth is proposed in Figure 7. In this model, X binds to and represses the action of the growth stimulator Y. Phosphorylation of X by Pi CDPK1 leads to release of Y (regulatory step 1, Figure 7); hence, excess Pi CDPK1 would lead to loss of polarity. Pi CDPK1-DN could potentially cause a loss of polarity by binding to and sequestering a fraction of unbound X, thereby reducing the pool of inhibitor available to repress Y. It would be likely in this scenario that the loss of polarity induced by Pi CDPK1-DN would be less than that induced by the wild-type enzyme, as was observed experimentally (Figures 2A and 2B).

To explain the growth inhibition caused by Pi CDPK1-CA, it is necessary to invoke another component in this pathway. Following the scenario above, Pi CDPK1-CA should lead to phosphorylation and inactivation of the repressor X, causing release of the stimulator Y and loss of growth polarity. However, as seen in Figures 2E and 2F, Pi CDPK1-CA caused a dramatic inhibition of pollen tube length and width. The key difference between wild-type Pi CDPK1 and the CA mutant is that in the mutant, kinase activity is not constrained by the apical Ca^{2+} gradient. Without this gradient in activity, the kinase will no longer be able to impose spatial organization on elements of the tip growth machinery. \( \Delta N \)– Pi CDPK1 does not cause loss of polarity (Figure 4B), suggesting that the plasma membrane plays an integral role in these events and that the growth promoting activity of Y is likely to be plasma membrane associated. A possible explanation for the inhibitory activity of Pi CDPK1-CA is that there is additional regulation of Y by another factor (Z) (regulatory step 2, Figure 7), the activity or localization of which is confined to the cortical growth zone. Under these conditions when Pi CDPK1-CA is expressed, phosphorylation of X increases liberating Y. As this reaction is independent of \([\text{Ca}^{2+}]_c\), Y would be redistributed to the entire plasma membrane rather than being focused to the cortical growth zone, and the unregulated activity of the CA kinase is likely to deplete X-Y complexes throughout the cell. Though total free Y has increased, it is dissipated, resulting in a reduction in Y in the cortical growth zone (the only place where Z is present to activate it) and growth inhibition. While this model appears tortuous, it not only explains the phenotypes observed, but a signaling pathway implicated in the regulation of pollen tube growth polarity that comprises precisely such a set of components.

Rop/Rac GTPase isoforms have been well demonstrated to be critical regulators of pollen tube growth polarity (Lin and Yang, 1997; Kost et al., 1999; Li et al., 1999; Cheung et al., 2003). These GTPases are regulated in several ways and would fit into the scenario proposed above. Recruitment of Rop/Rac to the plasma membrane is critical to biological function (Lin et al., 1996; Kost et al., 1999; Li et al., 1999) and is mediated by guanine dissociation inhibitors (GDIs). At the plasma membrane, the GTPase activity of Rop/Rac is regulated by guanine exchange factors, which promote activation through exchange of GDP for GTP. Substituting these components into the above model, Pi CDPK1 might act to phosphorylate GDI (factor X), leading to release of Rop/Rac (factor Y) from GDI-bound complexes, potentiating recruitment of these GTPases to the plasma membrane and their activation by guanine exchange factors (factor Z).

Consistent with the above model, the loss of polarity observed on overexpressing Pi CDPK1 phenocopies that caused by Rop1 overexpression (Li et al., 1999). The finding of Estruch et al. (1994) that antisense oligonucleotides of a maize homolog of Pi CDPK1 inhibited pollen germination and tube growth is also consistent with this model, as knockdown of CDPK is predicted to lead to a reduction or loss of GTPase recruitment to the plasma membrane and, hence, growth inhibition. Phosphorylation of RhoGDI
by PKC has been reported to regulate release of Rho GTPase from Rho/RhoGDI complexes in animals (Mehta et al., 2001), and we have recently obtained evidence that Pi CDPK1 regulates a *Petunia* RhoGDI1 homolog in a similar manner (G.M. Yoon and A. McCubbin, unpublished data).

Rop/Rac has been suggested to be upstream of the transient elevation of [Ca\(^{2+}\)]\(_i\), at the pollen tube tip (Zheng and Yang, 2000). This being the case, Pi CDPK1 would also be a downstream effector of Rop/Rac. The existence of such a positive feedback loop involving Rop/Rac and [Ca\(^{2+}\)]\(_i\) has been proposed (Zheng and Yang, 2000) and would be consistent with the observation that Pi CDPK1 overexpression not only leads to loss of growth polarity but also elevation of [Ca\(^{2+}\)]\(_i\), at the tube tip. One mechanism by which Pi CDPK1 might indirectly regulate Ca\(^{2+}\) dynamics via the Rop/Rac pathway is through the actin cytoskeleton. There is mounting evidence that Rop/Rac regulates actin dynamics (Chen et al., 2003; Gu et al., 2003, 2005), and involvement of actin microfilaments in ion channel regulation has been established in animal cells (Negulyaev et al., 2000) and reported in plant cells, including pollen (Hwang et al., 1997; Liu and Luan, 1998; Wang et al., 2004).

Seemingly contrary to the data discussed above, although increasing the levels of GTP or [Ca\(^{2+}\)]\(_i\) stimulates endo/exocytosis in the apical region of growing *Agapanthus* pollen tubes, elevation of GTP does not significantly affect [Ca\(^{2+}\)]\(_i\) (Camacho and Malhó, 2003). This suggests that actin exocytosis is regulated in a concerted but differentiated manner by [Ca\(^{2+}\)]\(_i\) and GTPases (Camacho and Malhó, 2003) and implies that Rop/Rac GTPases are not upstream of Ca\(^{2+}\). A possible explanation is that the elevation of GTP affected additional pathways. Defining these pathways and their regulation will be an important area for further study.

The results presented show that CDPK isoforms play multiple distinct roles in pollen tube growth. Interaction with the Rop/Rac GTPase pathway provides one possible explanation for Pi CDPK1 regulation of growth polarity. The general approach of expressing catalytically modified CDPK constructs in vivo has not been widely used. The results presented here along with those previously published (McCubbin et al., 2004) suggest that this approach can provide valuable information concerning the function of at least some CDPK isoforms.

**METHODS**

**3’ RACE**

Total RNA was purified from pollen using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription, 3’ RACE amplification, and purification of PCR products were performed using the conditions and primers as described by McCubbin et al. (2004). Products were cloned into pGEM-T Easy (Promega).

**cDNA Library Screening**

Library filters were prepared using standard procedures (Sambrook et al., 1989). CDPK fragments generated by 3’ RACE were labeled separately and combined to screen two independent poly(A)⁺ *Petunia inflata* cDNA libraries constructed in λZapII (Stratagene), constructed using the Superscript Choice system for cDNA synthesis (Life Technologies) according to the manufacturer’s instructions. The number of recombinant clones screened for each library was approximately equal to the respective primary titer of each library (~2 × 10⁹). Probes were radiolabeled with [³²P] using the RTS RadPrime DNA labeling kit (Life Technologies) and hybridized to filters for 16 h in hybridization buffer (10% [v/v] dextran sulfate, 1 M NaCl, 0.1% [v/v] SDS, and 100 μg/ml herring sperm DNA) at 62°C. After hybridization, filters were washed 3 x 20 min with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% (v/v) SDS. Autoradiography was then performed at ~70°C with an intensifying screen. Positive plaques were excised and subjected to secondary screening. Hybridizing plaques were purified, and in vivo excision was performed following the manufacturer’s protocol.

**RNA Gel Blot Analysis**

Total RNA was extracted from 100 mg of each tissue type using TRizol reagent according to the manufacturer’s protocol. Fifteen micrograms of total RNA was separated on a formaldehyde gel and stained with ethidium bromide to verify equal loading. The RNA was then transferred to Biodyne B membrane (Life Technologies) for hybridization. DNA probes were synthesized from cloned 3’ RACE fragments of Pi CDPK1 and Pi CDPK2 using the RTS RadPrime DNA labeling kit and hybridized to filters for 16 h in hybridization buffer at 62°C. After hybridization, filters were washed sequentially with 2× SSC, 0.1% (v/v) SDS; 0.5× SSC, 0.1% (v/v) SDS; and 0.1× SSC, 0.1% (v/v) SDS at 62°C for 20 min per wash. Autoradiography was performed at ~70°C with an intensifying screen. Autoradiographs were scanned using a LIDE flatbed scanner (Canon USA).

**Construction of Plasmids for Pollen and Escherichia coli Expression**

All constructs were generated by PCR, cloned into pGEM-T Easy vector, and sequenced prior to cloning into vectors for expression in pollen or *E. coli*. The coding regions of each CDPK isoform were converted to encode Ncol at the 5’ end using primers PC1/NcoF (5’-CCATGGGAACTTTGTGT-3’) for Pi CDPK1 and PC2/NcoF (5’-CCATGGGAACAATCGATGT-3’) for Pi CDPK2. Vector primers were used as reverse primers (3’ EcoRI sites present in the adapters used for library construction were used for subsequent cloning steps). The DN constructs were generated by overlapping PCR using DN-F (5’-ATCTATGAAAYCAACTCACC-3’) and DN-R (5’-AGGTTGAGTTTCTATGAGAT-3’) to replace codons Asp-196 of Pi CDPK1 and Asp-196 of Pi CDPK2 with codons for Asn. CA constructs were generated by truncating each isoform at the junction between the kinase and autoinhibitory domains using forward primers PC1/NcoF and PC2/NcoF (as described above with the reverse primers PC1/K-Sac-R, 5’-GAGCTTGTACCTCGATGC-3’, for Pi CDPK1 and PC2/K-Sac-R, 5’-GAGCTGCTAATTGTG-3’, for Pi CDPK2). For full-length GFP fusion constructs, stop codons were removed and Ncol sites introduced using PC1/Nco-R (5’-CCATGGAAACATTTCGAC-3’) and PC2/Nco-R (5’-CCATGGAAATTTC-3’, for Pi CDPK2). For full-length GFP fusion constructs, stop codons were removed and Ncol sites introduced using PC1/K (5’-CCATGGGCTCTCGATCCAGAGG-3’) and Pi CDPK1 and PC2/K (5’-CCATGGGCTTTTGATGATG-3’, for Pi CDPK2). KN constructs were generated by deleting the six N-terminal amino acids and adding 5’ Ncol sites, using primers 5’-CCATGGCCCAAATCTAGGA-3’ for Pi CDPK1 and 5’-CCATGGCTTACAAAATGCA-3’ for Pi CDPK2. KN constructs were cloned into pRSET B expression vector (Invitrogen) between Ncol and EcoRI sites. The 3’ EcoRI sites were derived either from the adaptors used for library construction for full-length constructs, pGEM-T Easy for deletion constructs, or pBluescript KS⁺ (Stratagene) for GFP fusion constructs. Pollen expression constructs...
were generated in pBluescript KS+ using the pollen-specific promoter Lat52 (Twell et al., 1990). ProDpactGFP was generated by cloning the coding region of enhanced GFP (Clontech) between the Lat52 promoter and a nos terminator in pBluescript KS+. All GFP fusion constructs were subsequently generated by cloning appropriate fragments into an Ncol site at the junction between the Lat52 promoter and enhanced GFP to generate in-frame fusions. Standard recombinant DNA methodology was used in all cloning steps.

**Transient Expression in Pollen**

*P. inflata* pollen was collected from freshly dehisced anthers (10 flowers/bombardment) and suspended by gentle vortexing in 200 μL of pollen germination medium (0.01% w/v H2BO3, 0.02% w/v MgSO4, 0.07% w/v CaCl2, 15% w/v PEG-4000, and 2% w/v sucrose). The pollen then was spotted onto a 2.5-cm2 piece of positively charged nylon membrane in a 9-cm Petri dish.

Microprojectile bombardment was performed using a PDS-1000/He biolistic system (Bio-Rad). Gold particles (1.1 μm) were prepared according to the manufacturer’s protocol (Bio-Rad) using 2 μg of plasmid DNA/0.5 mg of particles. Cobombardments were achieved by coating particles with 2 μg of each plasmid construct. Bombardment was performed at 28-inches Hg chamber vacuum, 1100 psi rupture disk, 0.25-inch gap distance, and 1-inch particle travel distance. After bombardment, the pollen was washed from the nylon membrane into a Petri dish with 4 mL of pollen germination medium and cultured on an orbital shaker at 100 rpm for 4 h at 30°C.

**Preparation of Recombinant CDPK**

For preparation of recombinant CDPK, *E. coli* transformed with the appropriate plasmid was grown overnight in 0.5-liter cultures. Purification of the His-tagged protein was performed by sequential chromatography first using His-Select Nickel Resin (Sigma-Aldrich), followed by phenyl Sepharose (Sigma-Aldrich) as described by McCubbin et al. (2004). Purity of the final protein product was assessed using SDS-PAGE.

**Kinase and Protein Assays**

Protein kinase activity was determined by measuring the incorporation of [γ-32P]ATP into PLARTLSVAGLPKK (Syntide-2, a Ca2+-calmodulin-dependent protein kinase II substrate; Sigma-Aldrich). Substrate phosphorylation and autophosphorylation assays were conducted as described by Ritchie and Gilroy (1998). Protein concentration was determined by the method of Bradford (1976) using BSA as a standard and the Bio-Rad protein assay kit. Free calcium concentrations were set in kinase assays using an EGTA/Ca2+ buffer system. Specific Ca2+ concentrations were calculated using WebMaxC standard software (http://stanford.edu/~cpatton/webmaxc/webmaxC.s.htm; Bers et al., 1994).

**Analysis of Transformed Pollen Tubes**

Epifluorescence microscopy for GFP observation was performed using an Orthomat epifluorescence microscope (Leitz) with a ×40, dry, 0.7–numerical aperture Fluor objective. GFP fluorescence was visualized using 480-nm excitation, 500-nm dichroic mirror, and >530-nm emission. Images were captured using a Senys cool CCD camera (Photometrics).

Confocal images were obtained using a Zeiss 510 laser scanning microscope (488-nm excitation, 515- to 565-nm emission for GFP). All confocal images were analyzed using Metamorph v4.5 image analysis software (Molecular Devices) and processed using Adobe Photoshop v3.5 (Adobe Systems).

**Cytoplasmic Ca2+ Concentration Measurements**

Pollen tubes were microinjected with the fluorescent Ca2+-indicating dye Indo-1–dextran, and [Ca2+] was monitored by ratio imaging using a confocal microscope (model LSM 410; Carl Zeiss Microimaging) according to Bibikova et al. (1997).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ147913 (Pi CDPK1) and DQ147912 (Pi CDPK2).

**Supplemental Data**

The following material is available in the online version of this article.

**Supplemental Table 1.** Specific Activities of Purified CDPK Proteins Expressed in *E. coli*.

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# Calcium-Dependent Protein Kinase Isoforms in *Petunia* Have Distinct Functions in Pollen Tube Growth, Including Regulating Polarity

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